15.1.3 Venom-neutralising potency test

This test determines the effectiveness of the antivenom to neutralise the overall toxic activity (the combined effects of the venom components responsible for) of the snake venom(s) against which the antivenom is designed. The first section of the test, to determine the lethal activity of the venom, is called the Median Lethal Dose (LD_{50}) assay and usually utilises mice of 18-20 g weight range. For new venoms whose LD_{50} is unknown, it is recommended that a range dose-finding study, using one mouse per venom dose, is performed to avoid using excessive numbers of animals.

LD_{50} Range-Finding Test: Various venom doses are prepared using saline solution as diluent, and aliquots of 0.2 ml of each dose are injected, using one mouse per dose, by the intravenous route in the tail vein (or, alternatively, by the intraperitoneal route (using injection volumes of 0.5 ml). Deaths are recorded at 24 hours (intravenous test) or at 48 hours (intraperitoneal test). On the basis of this preliminary dose-finding experiment, a range of venom doses causing 0% to 100% lethality is established and thus narrows the range of venom doses required to formally estimate the toxic activity of the venom.

The Median Lethal Dose (LD_{50}) assay: Groups of 5-6 mice of a defined weight range are injected intravenously, in the tail vein, with 0.2 mL of solutions of varying doses of venom dissolved in saline solution. A minimum of 5 mice is the smallest number recommended for obtaining statistical significance. In some laboratories the LD_{50} is estimated by the intraperitoneal route using an injection volume of 0.5 mL. Deaths are recorded at 24 hr (for assays involving intravenous injections) or at 48 hr (intraperitoneal injections), and LD_{50} is estimated by Probit analysis (Finney, 1971), Spearman-Karber (WHO, 1981) or alternative procedures (such as non-parametic methods). One venom LD_{50} is defined as the minimal amount of venom causing death in 50% of the mice injected. The test to assess the neutralizing potency of an antivenom is called the Median Effective Dose (ED_{50}) assay. For a new antivenom, it is recommended that a preliminary range dose-finding procedure is performed, using one mouse per antivenom dose.

ED_{50} Range-Finding Test: The selected multiple of the venom LD_{50} (3-5 LD_{50}) is mixed with different doses of antivenom and incubated at 37°C for 30 minutes and each mixture injected into a single mouse. This preliminary test should establish a range of antivenom volumes that result in 100% survival and 100% death of the injected mice and thus narrows the range of doses required for the formal ED_{50} test.

The Median Effective Dose (ED_{50}) assay: This test involves the incubation of a fixed amount of venom ('challenge dose', usually corresponding to three to five LD_{50}s), with various volumes of the antivenom adjusted to a constant final volume with saline solution (WHO, 1990; Theakston et al., 2003; Rojas et al., 2005). The mixtures are incubated for 30 minutes at 37 °C, and then aliquots of 0.2 mL of each mixture are injected into groups of 5-6 mice of a defined weight range by the intravenous route, using the tail vein. A control group injected with a mixture of the venom 'challenge dose' with saline solution alone (no antivenom) should be included to confirm that the venom 'challenge dose' induces 100% lethality. When the test is performed by the intraperitoneal route, a volume of 0.5 mL is administered. Centrifugation of the

antivenom-venom mixtures is not recommended because residual venom toxicity may remain in the immunoprecipitates. After injection, deaths are recorded at 24 hours (intravenous injection) or at 48 hours (intraperitoneal injections) and the results analysed using Probit analysis (Finney, 1971), Spearman-Karber (WHO, 1981) or alternative procedures (such as non-parametic methods). The Median Effective Dose (ED_{50}) of an antivenom is defined as the volume of antivenom that protects 50% of the mice injected.

The ED_{50} can be expressed in various ways: (a) mg of venom neutralized by mL of antivenom; (b) µL antivenom required to neutralize the 'challenge dose' of venom used; (c) µL of antivenom required to neutralize one mg of venom; and (d) number of LD_{50}s of venom neutralized per mL of antivenom. Every production laboratory and every national regulatory agency should establish the accepted levels of neutralizing potency for the various antivenoms being produced and distributed. In this regard, it is important to guarantee that a standardized assay is used by the manufacturing laboratories. Since the methodology to estimate antivenom potency (ie, ED_{50}) varies between laboratories and countries, manufacturers should disclose the conditions in which the potency of their antivenoms is estimated to the correspondent regulatory agencies in the course of their licensing and control procedures.

The protocols for the selection and quality control of the venoms used for these potency assays should be established in each quality control laboratory (see Section 8). Venoms used in this test should correspond to a representative pool obtained from at least 25-50 well-identified snake specimens collected from various regions within the geographical range of distribution of the species in a country. These national reference venom pools must be evaluated periodically in order to assure that they have not deteriorated (see Section 8 on quality control of venoms).

Until *in vitro* or alternate tests of lesser severity become accepted, these venom $LD_{(50)}$ and antivenom $ED_{(50)}$ assays should be performed by all manufacturers before an antivenom can be used in humans. The assays should be conducted under conditions causing the minimal possible suffering to the experimental animals.

15.1.4 Osmolality

Osmolality can be measured to determine the tonicity of the antivenoms. It is recommended that it be more than 240 mosmom/kg. Determination of osmolality is also an indirect means to determine the quantity of salts or excipients added for formulating the batch.

15.1.5 Identity test

When several types of antivenoms are produced by a single laboratory, the identity of each batch of antivenom should be controlled. Identity tests may include biological assays as well as physico-chemical and immunological tests. Double immunodiffusion assays, confronting the antivenom with the venoms against which the antivenom is designed, are often used. In the case of laboratories that use various animal species to raise antivenoms, i.e. horses and sheep, an

immunological identity test should be used to identity the mammalian species in which the antivenoms are produced. The potency assay against venoms is another way to identify antivenoms.

15.1.6 Protein concentration

The total protein concentration of antivenoms is performed using the Kjeldahl method for nitrogen determination. Alternatively, several colorimetric procedures can be used, measuring absorbance at 280 nm. The presence of preservatives should be taken into account since they may interfere with some protein determination methods (Tuck and Ng, 2005).

The total concentration of proteins in antivenoms should not exceed 10 g/dL, unless a higher protein content is justified and authorised by the competent authority.

15.1.7 Purity

The purity of the active substance, i.e. intact immunoglobulin or immunoglobulin fragments, should be assessed., They should constitute the great majority of the preparation, ideally greater than 90%.

Electrophoretic methods in polyacrylamide gels (SDS-PAGE run under reducing or non-reducing conditions) are suitable for this purpose, since these techniques allow the detection and monitoring of IgG, $F(ab')_2$, Fab, non-IgG plasma protein contaminants (in particular albumin), and degradation products. The electrophoretic pattern should be compared to that of a reference preparation. A semi-quantification can be performed by calibration of the procedure. Of particular relevance is the assessment of the albumin content which ideally should not exceed 1 % ot total protein content. The following approach can serve as a guide in assessing the purity of antivenoms:

SDS-PAGE under non-reducing conditions. This analysis can inform qualitatively (or, at best, semi-quantitatively) on the amounts of intact immunoglobulins, digestion products and, importantly, on the presence of high molecular weight oligomers (soluble aggregates) and low molecular mass contaminants (which are expected in the case of enzymatically-digested antivenoms).

SDS-PAGE under reducing conditions. Analysis under these conditions can inform on the amount of immunoglobulins and their fragments by direct visualization of intact and/or digested immunoglobulin heavy chains.

15.1.8 Molecular-size distribution

The presence of aggregates (which can be reactogenic) and other components in antivenoms can be assessed by size-exclusion liquid chromatography (gel filtration) in HPLC or FPLC systems.

Densitometric analyses of chromatographic profiles allow the quantification of protein aggregates and of the relative abundances of: intact immunoglobulins, divalent immunoglobulin fragments (F(ab')2), monovalent immunoglobulin fragments (Fab), dimers, as well as low molecular mass enzymatic digestion products.

In intact immunoglobulin-based antivenoms this method allows quantitation of albumin as its molecular mass (~66 kDa) can be resolved from the ~160 kDa peak of intact immunoglobulins.

15.1.9 Pyrogen test

Antivenoms should comply with the rabbit pyrogen test where required by the local regulations. This test is based on intravenous injection of antivenoms in the ear vein of rabbits (usually 1.0 to 3.0 mL per kg body weight), followed by the measurement of rectal temperature at various time intervals after injection. The detailed procedures are described in various Pharmacopeias.

Bacterial lipopolysaccharides can also be detected by the *Limulus* amebocyte lysate (LAL) test. The test should be validated for each type of antivenom, since there have been reports of false positive and false negative reactions when testing antivenoms and other plasma-derived products. The sensitivity of this LAL test should be correlated with the rabbit pyrogen test, and the endotoxin limits established. When regulation allows, a validated LAL test is used in place of the rabbit pyrogen test.

15.1.10 Abnormal toxicity test

The abnormal toxicity test is increasingly abandoned in most regulations as it provides limited information for routine quality assessment of a product. Correct implementation of Good Manufacturing Practices should provide evidence that the product would comply with the test for abnormal toxicity.

15.1.11 Sterility test

Antivenoms should be free of bacteria and fungi, i.e. should be sterile. The sterility test is performed following methodologies specified in various Pharmacopoeias such as the European Pharmacopoeia.

Since antivenoms may contain preservatives in their formulation, it is necessary to neutralize the preservatives before the samples are added to culture media. This is usually performed by filtering a volume of antivenom through a 0.45 um pore membrane, and then filtering through the same membrane a solution that neutralizes the bacteriostatic and fungistatic effects of the preservatives used in antivenom. The membrane is then aseptically removed and cut into two halves. One half is added to trypticase soy broth and the other is added to thioglycolate medium. Control culture flasks are included for each medium. Flasks are incubated at 20-25 °C (trypticase soy broth) or at 30-35 °C (thioglycolate) for 14 days. Culture flasks are examined daily for bacterial or fungal growth. The number of vials tested per batch should be in compliance with local regulations.

15.1.12 Concentration of sodium chloride and other excipients

The concentration of the various excipients or stabilizers added for formulation should be determined using appropriate chemical methods.

15.1.13 Determination of pH

The pH of antivenom should be determined, using a potentiometer.

15.1.14 Concentration of preservatives

When used in the formulation of antivenoms, the concentration of preservatives (phenols, cresols) should be quantified. The acceptable range of preservative concentration in antivenoms should be established and validated in each quality control laboratory. Phenol concentration should not exceed 2.5 g/L and metacresol 3.5 g/L.

Phenol concentration can be determined on the basis of the reactivity of phenol with 4-aminoantipyrine, under alkaline conditions (pH 9.0-9.2) in the presence of potassium ferrocyanide as oxidant. A colour product is formed whose absorbance is recorded at 495 nm. Other methods are also available. Cresol can be determined by HPLC methods.

15.1.15 Agents used in plasma fractionation (ammonium sulphate, caprylic acid, enzymes)

The chemical reagents used in the precipitation and purification of antivenoms, such as ammonium sulphate, caprylic acid and others, should be removed from the final product during diafiltration or dialysis. Limits should be established and their residual amount quantified in the final product. Likewise, the elimination of pepsin or papain from the final preparations should be

guaranteed, especially for preparations that are maintained liquid in order to avoid proteolytic activity that may damage the antivenoms.

The determination of the residual amount of agents used in plasma fractionation could be excluded from routine release test the process of manufacturing has been validated to eliminate these reagents. The detection of residual reagents can also be performed on the final bulk rather than in the final product.

15.1.16 Visual inspection

All the vials or ampoules of each batch of liquid antivenoms should be inspected, either visually or using a mechanical devices. Any vial or ampoule presenting turbidity, abnormal coloration, presence of particulate matter, or defects of the vial, stopper, or capsule should be discarded. In the case of freeze-dried products, a representative sample of the whole batch should be dissolved in the solvent and inspected as described. Turbidity can be assessed quantitatively by using a turbidimeter.

15.1.17 Residual moisture (freeze-dried preparations)

Residual moisture content can be determined by several methodologies, such as (a) a gravimetric method assessing the loss of weight on heating, (b) the Karl-Fischer titration, based on the principle that iodine, together with pyridine, sulphur dioxide and methanol from the reagent react quantitatively with water, and (c) thermogravimetric methods. The methodology most commonly recommended is the Karl-Fischer titration. Every manufacturing and quality control laboratory must establish the accepted maximum residual moisture for their antivenom. Residual moisture should be less than 3%.

15.2 Antivenom reference preparations

The use of internal in-house reference preparations of antivenoms is recommended, instead of international standards, since the issues of potency, specificity, and purity can only be compared with antivenoms of similar specificity and neutralizing profile. An in-house reference preparation should be obtained from a suitable batch of the product that has been fully evaluated by the quality control laboratory. For assays not related to potency or specificity, such as the quantification of proteins, preservatives and excipients, international standards can be used.

15.3 Main recommendations

- **Quality control of antivenom preparations, both for product intermediates and final product, as part of the batch release, should be performed by the manufacturers. National regulatory agencies will review the tests performed by the manufacturer and select which tests to develop when required, on a case by case basis.**
- **Quality control tests to be performed by manufacturers as part of the batch release include: neutralization potency test against the most relevant venoms to be neutralized, identity test, protein concentration, purity of the active substance, content of protein aggregates and non-IgG contaminants, pyrogen test, sterility test, concentration of excipients, osmolality, pH, concentration of preservatives, determination of traces of agents used in plasma fractionation, visual inspection, and, for freeze-dried preparations, residual moisture and solubility.**
- **Antivenom reference preparations reflecting specific characteristics of antivenoms produced should be prepared by each manufacturer to be used as standards in their laboratory settings. in particular to measure neutralization capacity of their specific antivenom products against targeted venoms,**

16 STABILITY, STORAGE AND DISTRIBUTION OF ANTIVENOMS

16.1 Stability

Real-time stability studies should be performed to determine the stability of antivenoms. These studies should be done when a new product, a process change, or a new formulation is developed. They are essential to define the shelf-life of the product and are intended to prove that the antivenom remains stable and efficacious until the expiry date.

It was considered in the past that liquid preparations have a shelf-life up to 3 years at +2-+8 °C, and freeze-dried preparations up to 5 years in the dark at room temperature, Nevertheless, the actual stability of each antivenom formulation should be appropriately determined by each manufacturer. It is highly recommended that manufacturers perform stability studies to evaluate the possibility of their preparations to be stored for a long period of time (e.g. up to 10 years) under non-refrigeration (for instance at 30°C).

Real-time stability tests should be performed under the expected storage conditions of the antivenom. In addition, these tests could be performed under worst case conditions of storage. Quality control parameters are determined at regular pre-established time-intervals. Essential parameters, among others, include venom neutralization potency, turbidity and content of aggregates, since those are especially prone to evolve upon storage.

Accelerated stability studies are not a substitute for real-time data but may be performed to provide early useful information on the product stability profile. The antivenom is exposed to harsher conditions than usual, such as higher temperature, and the stability is assessed over a shorter time span. Accelerated stability tests do not always correlate with real time tests.

16.2 Storage

Antivenoms should be stored at the temperature range that assures stability, as found by stability tests. This is particularly critical for liquid formulations, which usually require 2-8 °C storage. Therefore, deviations from this temperature range, due to interruptions in the cold chain during transportation or storage, are likely to result in product deterioration. The design of adequate cold chain programs, as part of the public health systems in every country, is highly critical, and national protocols should be developed. The distribution policies for national vaccination programmes can be used for the transportation and storage of antivenoms. The stability of liquid preparations at temperatures higher than 2-8 °C should be evaluated and, if needed, new formulation allowing such storage conditions should be developed.

16.3 Distribution

The adequate distribution of antivenoms is a matter of great concern in many regions of the world. Since most antivenoms available are liquid preparations, the maintenance of an adequate cold chain must be guaranteed, despite the difficulties to be encountered in rural areas of some developing countries. National and regional health authorities should develop distribution strategies to ensure that antivenoms are allocated to the areas where they are needed or use the distribution channels in place for other national primary health care programmes. This consideration includes both the specificity of the antivenom and the number of vials or ampoules to be distributed. This is particularly relevant in countries that use monospecific antivenoms, since distribution of these products should be guided by the known distribution of the species. In order to ensure appropriate supply for clinical use, inventories should be in excess of the estimated number of cases to allow for unpredictable surges in local demand, accepting the fact that some antivenoms would be left unused at the time of expiry date.

16.4 Main recommendations

- **The quality control of each antivenom batch prepared by a manufacturer should include the potency test for neutralization of lethality (ED_{50})**
- **In general, liquid preparations require a cold chain, whereas freeze-dried preparations do not. However, storage conditions are product/formulation-specific**

and may vary. Manufacturers should therefore determine the stability of each antivenom pharmaceutical preparation by conducting real-time stability studies.

- **Manufacturers should study the stability of antivenoms at the ambient temperatures where the product will be used**
- **The distribution of antivenoms by health authorities should rely on a proper assessment of the epidemiology of snakebite envenomings, and on the proper knowledge of the geographical distribution of the most relevant venomous species. This is particularly important for monospecific antivenoms.**
- **NRAs should ask manufacturers to provide preclinical assessment information of all antivenom used in their territories against the venoms found in the region/country where the product is intended to be used.**

17 PRECLINICAL ASSESSMENT OF ANTIVENOMS

17.1 Introduction

A fundamental and ethical requirement of all new therapeutic agents intended for human use is that their safety and efficacy should be established, initially by preclinical *in vitro* and *in vivo* laboratory tests and, if the results of these prove satisfactory, by clinical trials in human patients. Information supporting the physicochemical characterization of the new antivenom, such as . protein content and level of purity of the preparation should be available before clinical studoes are initiated. The assays to be performed are described under Section 15, on quality control of antivenoms, should be considered.

Preclinical testing of antivenoms should be implemented when: (a) a new antivenom is being developed, (b) an existing antivenom is to be introduced for use in a new geographical region or country. In both cases, preclinical studies in animal models should be a regulatory requirement enforced by the medicines regulatory authorities as part of the licensing procedures of antivenoms.

The preclinical tests of new or existing antivenoms necessitate the use of experimental rodents. Despite reservations over the physiological relevance of these animal models to human envenoming and the severity of these *in vivo* assays (Section 17.4 & 17.5), the tests for determining venom lethality (Median Lethal Dose, LD_{50}) and antivenom neutralizing capacity (Median Effective Dose, ED_{50}) are currently the only validated means of assessing venom toxicity and antivenom neutralizing potency by both manufacturers and regulatory authorities worldwide.

It is important to make a distinction between 'essential' and 'recommended' preclinical assays. The 'essential' preclinical assays consist of the overall evaluation of toxic activity of the specific snake venoms (LD_{50}) and the correspondent antivenom neutralising efficacy of the overall

venom(s) toxicity (ED_{50}). These tests are required (i) as a routine quality control of antivenom potency, (ii) to test the ability of a new antivenom to neutralise the venoms from snakes from the country or region where it is going to be introduced, (iii) to show neutralising efficacy of an existing antivenom against medically relevant species in a new geographical region or country. In summary, any antivenom used therapeutically in a given region or country should have been preclinically assessed using the 'essential' assays against the relevant snake venoms before the product is used in humans.

Preclinical testing of antivenoms includes also a number of assays whose selection depends on the main pathophysiological effects induced by the venom to be tested. Additional tests are therefore strongly recommended for new antivenoms and for new applications of existing antivenoms to determine whether they are effective in eliminating the most clinically-relevant pathophysiological effects induced by the specific venom(s)of interest.

As an example, a new antivenom developed against *Echis ocellatus* envenoming should be tested for its preclinical neutralising potency (LD_{50} and ED_{50} tests) (i) before it is released for the first time for human trials and (ii) as a routine quality control of the potency of subsequent batches. It is also recommended that the first batch be preclinically tested for its ability to eliminate venom induced coagulopathy and haemorrhage – the most medically-important effects of *E. ocellatus* envenoming.

17.2 Essential assay for preclinical testing of antivenoms: Prevention of lethality

The methodology for estimating the Median Lethal Dose (LD_{50}) of venoms and the Median Effective Dose (ED_{50}) of antivenoms is described in detail in the section on Quality Control of antivenoms (Section 15). The same methods used in the routine quality control of antivenoms should be used in the preclinical testing of all new antivenoms and all new applications of existing antivenoms.

17.3 Additional recommended assays for preclinical testing of antivenoms

It is necessary to test whether antivenoms are effective in the neutralization of the most relevant pathophysiological effects induced by a particular venom. These 'recommended' preclinical tests are however not intended for the routine quality control of antivenom batches. The relevant methods to be used are listed below.

17.3.1 Neutralization of venom haemorrhagic activity

Many venoms, especially those of vipers, exert powerful local and systemic haemorrhagic activity which is due primarily to venom zinc metalloproteinases. These enzymes damage the

basement membrane that surrounds endothelial cells of capillary blood vessels resulting in bleeding into the tissues. Bleeding into the brain and other major organs is considered to be the major lethal effect of envenoming by many viperid species (Reid and Theakston, 1983). The minimum haemorrhagic dose of a venom (MHD) is defined as the amount of venom (in µg dry weight) which, when injected intradermally, induces in mice a 10 mm haemorrhagic lesion 24 hr after injection (Theakston and Reid, 1983; Gutiérrez et al., 1985).

The MHD test is carried out by preparing aliquots of 50 µl of physiological saline solution containing a range of venom doses. Mice (18-20 g body weight/ 5 mice per group) are placed under light general anaesthesia (eg, halothane/oxygen) and the hair surrounding the injection site shaved. The venom solutions (50 µl) are injected intradermally in the shaved skin. After 24 hours, mice are killed using an approved humane procedure, the area of the injected skin is removed, and the haemorrhagic lesion in the inner side of the skin is measured using calipers in two directions with background illumination. Care should be taken not to stretch the skin. The mean diameter of the haemorrhagic lesion is calculated for each venom dose and the MHD estimated by plotting mean lesion diameter against venom dose and reading off the dose corresponding to a 10 mm diameter (Theakston and Reid, 1983; Gutiérrez et al., 1985).

To estimate the ability of an antivenom to neutralise venom-induced haemorrhage, a 'challenge dose' of venom is selected, which corresponds to one or multiple MHDs. Between one and five MHDs have been used as the 'challenge dose' by different laboratories. The test is carried out as above, using 5 mice per group. Mixtures of a fixed amount of venom and various dilutions of antivenom are prepared so that the 'challenge dose' of venom is contained in 50 µl. Controls must include venom solutions incubated with physiological saline solution alone. Mixtures are incubated at 37 °C for 30 min, and aliquots of 50 µl are injected intradermally in lightly anaesthetised mice. The diameter of haemorrhagic lesions is quantified as described above, and the neutralising ability of antivenom, expressed as MHD-Median Effective Dose (ED_{50}), is estimated as the volume of antivenom, in microliters, which reduces the diameter of haemorrhagic lesions by 50% when compared with the diameter of the lesion in animals injected with the control venom/saline mixture (Gutiérrez et al., 1985).

17.3.2 Neutralization of venom necrotising activity

Venom-induced local dermonecrosis is a major problem in human victims of snakebite and it has long been considered important to have an assay system to evaluate the effect of an antivenom on this pathology. However, it should be stated that the value of antivenoms in overcoming the cytolytic effects of venoms has not yet been established; indeed, there is considerable doubt whether antivenom is useful in obviating such effects in human victims of snakebite. This is because venom-induced dermonecrosis occurs quickly after a bite and there is usually a considerable delay between the envenoming of a victim and their arrival in hospital for treatment. Consequently, antivenom therapy can have little or no effect in reversing the damage (Reid, 1964; Gutiérrez et al., 1998). Animal experiments in which the antivenom was administered to the animal at different times after the antivenom support this consideration (Iddon et al., 1987; Gutiérrez et al., 1998).

The minimum necrotizing dose (MND) of a venom is defined as the least amount of venom (in µg dry weight) which, when injected intradermally into groups of five lightly anaesthetised mice (18-20 g body weight), results in a necrotic lesion of 5 mm diameter 3 days later. The method used is the same as that for the MHD, except that the skin is examined 3 days after the intradermal injection of the venom (Theakston and Reid, 1983).

To estimate the ability of an antivenom to neutralise venom-induced dermonecrosis, a 'challenge dose' of venom is selected, usually between one and two MNDs. The test is carried out as above, using 5 mice per group. Mixtures of a fixed concentration of venom and various dilutions of antivenom are prepared so that the venom 'challenge dose' is contained in 50 µl. Controls include venom solutions incubated with physiological saline solution alone. Mixtures are incubated at 37 °C for 30 min, and aliquots of 50 µl are injected intradermally in lightly anaesthetised mice (Laing et al., 1992; Theakston, 1986). The diameter of dermonecrotic lesions is quantified 3 days after injection, as described above, and the neutralising ability of antivenom, expressed as MND-Median Effective Dose (ED_{50}), is estimated as the volume of antivenom, in microlitres, which reduces the diameter of necrotic lesions by 50% when compared with the diameter of the lesion in mice injected with the control venom/saline mixture.

17.3.3 Neutralization of venom procoagulant effect

Many venoms, especially from some vipers, cause consumption of coagulation factors which results in incoagulable blood. This, combined with the haemorrhagic nature of some of those venoms, can result in a very poor prognosis for a severely envenomed patient. Simple *in vitro* methods exist to measure this venom-induced pathophysiological effect and the ability of an antivenom to eliminate it.

The minimum coagulant dose (MCD) of a venom is defined as the least amount of venom (in mg dry weight per litre of test solution or µg/ml) that clots either a solution of bovine fibrinogen (2 g/litre) in 60 sec at 37 °C (MCD-F) and/or a standard citrated solution of human plasma (fibrinogen content 2.8 g/litre) under the same conditions (MCD-P).

For measurement of the MCD-F, 50 µl of physiological saline with final venom concentrations ranging from 240 to 0.5 mg/litre is added to 0.2 ml of bovine fibrinogen solution at 37°C in new glass clotting tubes. The solutions are mixed thoroughly and the clotting time recorded. The MCD-P is estimated by adding the same venom concentrations to 0.2 ml of the standard human plasma solution under identical conditions and recording the clotting time. In each case, the MCD is calculated by plotting clotting time against venom concentration and reading off the level at the 60 second clotting time (Theakston and Reid, 1983).

To estimate the ability of an antivenom to neutralise venom procoagulant activity, a 'challenge dose' of venom is selected, which corresponds to one MCD-P or one MCD-F. Mixtures of a fixed concentration of venom and various dilutions of antivenom are prepared so that the 'challenge dose' of venom is contained in 50 µl. Controls include venom solutions incubated with physiological saline solution alone. Mixtures are incubated at 37 °C for 30 min, and aliquots of 50 µl are added to 0.2 ml of plasma or fibrinogen solution, as described. The formation or

absence of clots is observed during a maximum of 30 min. The minimum volume of antivenom which completely prevents clotting is estimated and corresponds to the MCD-Effective Dose.

17.3.4 Neutralization of in vivo *venom defibrinogenating activity*

This test is a direct measure of the *in vivo* defibrinogenating effect of certain venoms.To measure the minimum venom defibrinogenating dose (MDD), a wide range of venom doses is selected and each dose, in a volume of 0.2 ml, is injected intravenously into 4 mice (18-20 g body weight). One hour after injection, the mice are placed under terminal general anaesthesia and bled by cardiac puncture. The blood from each animal is placed in a new glass clotting tube, left at room temperature for one hour and the presence/absence of a clot recorded. The MDD is defined as the minimum dose of venom that produces incoagulable blood in all mice tested within one hour of intravenous injection.

Antivenom neutralisation of the venom component(s) responsible for *in vivo* defibrinogenation is estimated by incubating a 'challenge dose' of venom, corresponding to one MDD, with different amounts of the antivenom. Controls should include venom solutions incubated with saline solution instead of antivenom. Mixtures are incubated at 37 °C for 30 min before injection of 0.2 ml by the intravenous route in groups of 4 mice (18-20 g body weight). After one hour, mice are bled as described above, and blood placed in new glass clotting tubes and left undisturbed for one hour at room temperature, after which the presence/absence of clot is recorded. Neutralising ability of antivenoms is expressed as MDD-Effective Dose, corresponding to the minimum volume of antivenom in which the blood samples of all injected mice showed clot formation (Theakston, 1986; Gené et al., 1989).

17.3.5 Neutralization of venom myotoxic activity

The presence of myotoxic components in a venom results in the degeneration of skeletal muscle by breaking down muscle fibres. Damage is characterised by the disruption of plasma membranes, local infiltration of inflammatory cells and oedema. Myotoxicity is characterised by the appearance of myoglobin in urine and by increments in the serum levels of muscle-derived enzymes, such as creatine kinase (CK). Myotoxic phospholipase A_2 (PLA_2) enzymes are found in a wide range of snake venoms. Some of these PLA_2s may be primarily myotoxic, or neurotoxic, or both. In addition, myotoxicity may occur as a consequence of ischaemia induced in muscle fibres by the effect of haemorrhagic venom components in the microvasculature (Gutiérrez et al., 1995).

Venom myotoxic activity is determined by injecting rats or mice with various doses of venom in a constant volume of 50 µl (using saline solution as diluent) into the right gastrocnemius muscle. In the case of mice, groups of 5 animals of 18-20 g body weight are used per dose. Control animals are injected with the same volume of saline solution. Tail-snip blood samples are collected at a specific time interval (3 hr in mice), and the CK activity of serum or plasma is determined using commercially-available diagnostic kits (Gutiérrez et al., 1992; Alam et al.,

2002). Myotoxic activity is expressed as the Minimum Myotoxic Dose (MMD), defined as the amount of venom that induces an increment in serum/plasma CK activity corresponding to four times the activity in serum/plasma of animals injected with saline solution alone. Myotoxicity can also be assessed by histological evaluation of muscle damage after venom injection, although this is a more expensive and more time consuming method than the CK determination.

To estimate the ability of an antivenom to neutralise venom myotoxicity, a 'challenge dose' of venom is selected, which corresponds to 3 MMDs. The test is carried out as above, using 5 mice per group. Mixtures of a fixed concentration of venom and various dilutions of antivenom are prepared so that the 'challenge dose' of venom is contained in 50 µl. Controls include venom solutions incubated with physiological saline solution alone. Mixtures are incubated at 37 °C for 30 min, and aliquots of 50 µl are injected into the gastrocnemius muscle, as described above. Blood samples are collected 3 hours after injection (in the case of mice) and serum/plasma CK activity is quantified. The neutralising ability of antivenom, expressed as MMD-Median Effective Dose (ED_{50}) is estimated as the volume of antivenom, in microliters, which reduces the serum/plasma CK activity by 50% when compared to the activity of animals injected with venom incubated with saline solution only (Rojas et al., 2005).

17.3.6 Neutralization of venom neurotoxic activity

Several laboratory methods for assessing venom-induced neurotoxicity have been developed (e.g. chick biventer cervicis nerve-muscle preparation (Ginsberg and Warriner, 1960; Harvey et al., 1994); mouse hemidiaphragm phrenic nerve preparation (Bulbring, 1946; Kitchen, 1984; Jones et al., 1999; Crachi et al., 1999; Sells, 2003), but they are difficult to perform, require costly equipment and expert technological help and are unlikely to be practicable for most antivenom producers. Mouse lethality tests are usually reliable in predicting the neutralisation of neurotoxic effects of venoms.

17.4 Development of alternative assays to replace murine lethality testing

In vivo murine assays cause considerable suffering and there have been calls for the development of alternate assays to replace the standard LD_{50} and ED_{50} tests. The controversy relates to the balance between (i) the clinical benefit to humans of preclinical testing and (ii) the cost to the experimental rodents (death, pain and distress). This issue is of considerable concern and *in vivo* tests should be conducted with the minimal number of animals necessary and using protocols designed to minimise pain and suffering. There are alternative tests (Sells, 2003) which reduce the need for experimental animals, use alternative non-sentient systems or utilise *in vitro* test systems. Unfortunately, such systems cannot currently replace the rodent toxicity tests. Consequently, the development of alternative methods to animal testing in the preclinical evaluation of antivenoms, should be encouraged and when live animals are absolutely necessary, anaesthesia or analgesia should be (i) considered and (ii) evaluated to ensure that the humane

benefits of anaesthesia or analgesia to the experimental animals do not invalidate the objectives of the assay by altering relevant physiological processes (Theakston et al., 2003). The establishment of humane end points to reduce suffering and limiting the duration of the assays to reduce the period of animal suffering is also encouraged but also need to be carefully evaluated to ensure the validity of the results.

17.5 Limitations of preclinical assays

It is acknowledged that the *in vivo* and *in vitro* 'essential' and 'recommended' preclinical tests have physiological limitations (the venom and venom/antivenom injection protocols do not represent the natural situation, and rodent physiological responses to envenoming and treatment may differ from humans) that make the rodent model of human envenoming and treatment less than ideal. Care therefore should be taken to avoid simplistic extrapolations from this assay to the clinical situation. Nevertheless, the LD_{50} and ED_{50} tests represent the methods most widely used for assessment of antivenom potency, and a number of clinical trials have demonstrated that the ED_{50} test is useful (Theakston et al., 1995; Sells, 2003), but not infallible (Keegan et al., 1964, Warrell et al., 1980), at predicting the efficacy of antivenoms in the clinical setting. An additional value of these tests is the assurance that antivenoms are manufactured with an accepted, quantifiable and uniform neutralizing potency.

17.6 Main recommendations

- **Preclinical testing of antivenoms both to determine the purification profile of the preparation as well as its venom(s) neutralization capacity in animal models should be a minimum regulatory requirement to be enforced by the medicines regulatory**
- **The estimation of the ability of an antivenom to neutralize the lethal activity of venom(s) (LD_{50} and ED_{50}) is the most relevant preclinical assessment and should be performed for all antivenoms.**
- **All new antivenoms as well as existing antivenoms to be used in new geographical areas should furthermore be assessed for their ability to eliminate specific pathologies exerted by the venoms of the snakes for which the antivenom has been designed. The selection of which preclinical 'recommended' test(s) to perform will depend on the predominant pathophysiological effects induced by the specific snake venom and be appropriately adapted for each antivenom. The 'recommended' tests are not required for quality control assessment of subsequent batches of antivenom.**
- **Pre-clinical testing still relies heavily on the use of laboratory rodents and involves an unsatisfactorily high degree of suffering. The working protocols should recommend anaesthesia and analgesia to reduce suffering, where possible. Animals should be housed, fed and handled according to approved veterinary standards.**

- **Research should be promoted for the development of (i) refinements of the *in vivo* assay protocols to reduce pain and suffering of animals, and (ii) of *in vitro* alternatives to the *in vivo* assays to reduce the number of animals used in preclinical testing. Results of any modified *in vivo*, or new *in vitro* protocols, should be rigorously compared with results from existing protocols to ensure the statistical validity of the new developments.**

18 CLINICAL ASSESSMENT OF ANTIVENOMS

18.1 Introduction

Antivenoms are virtually unique among pharmaceutical agents in that they have been used in human patients for more than 100 years with little attention to clinical trials of their efficacy and safety. However, since the 1970s it has been clearly demonstrated that it is possible to carry out dose-finding and randomised controlled (comparative) trials in human snakebite victims. These studies have yielded very valuable information as in the case of clinical trials of other therapeutic agents which are generally regarded as the essential basis for regulatory approval.

There are well established pathways for the evaluation of new therapeutic products.

- Phase I: healthy volunteer studies - safety (frequent and severe adverse reactions)
- Phase II: limited efficacy and reactogenicity studies, often dose finding
- Phase III: full scale clinical evaluation, often randomised controlled trials
- Phase IV: post-marketing surveillance

The appropriateness of this pathway for antivenoms depends upon a number of factors, including whether an antivenom is new or has been previously used in another setting, the practicality of undertaking such studies as well as national regulatory considerations.

18.1.1 Phase I studies

Conventional clinical studies using healthy volunteers are rarely appropriate in the case of antivenoms. Phase 1 studies are primarily designed to detect unanticipated adverse events and there is extensive experience with antivenom treatment that allows a basic understanding of of its pharmacokinetics and of risks of adverse reactions due to poor immunoglobulin purification process. Furthermore, preclinical studies, that should be performed before use of an antivenom in humans, such as biochemical characterization or experimental evaluation of purity, can provide a reliable indication of the allergic or pyrogenic risks. Therefore, there is rarely an indication for phase 1 studies of antivenom.

18.1.2 Phase II/III studies

Phase II studies are usually conducted to establish safety of a product, give an indication of efficacy and optimize doses. Phase III studies are normally used to confirm efficacy of a product and study reactogenicity in a larger number of patients, often in comparison with an existing product, the efficacy and reactogenicity of which was previously assessed. The use of a placebo, although it may give a more reliable response, is generally regarded as unethical. The conduct of such studies is guided by the principles set down in the international regulations governing Good Clinical Practice (ICH-GCP; WHO, 1995; WHO 2005). These principles emphasise the responsibilities of the researcher and of the organisation sponsoring the research, act to protect participants in research and ensure that the conduct of the trial is likely to lead to reliable results.

18.2 Antivenom clinical studies

Although pre-clinical testing may be valuable in ensuring that antivenoms neutralise the venoms of interest, the complex effects of venom in humans and the need to consider pharmacokinetics means that, ultimately, the efficacy and safety of antivenoms for the treatment of human envenoming needs to be determined by well designed clinical studies. Clinical studies of antivenoms primarily address three main issues:

- Assessment of efficacy of the antivenom in patients
- Assessment of the safety of an antivenom (primarily reaction rates)
- Optimisation of antivenom dose

The reaction rates for a given antivenom preparation are unlikely to vary between geographical settings. However, both efficacy and dose-finding studies may need to be repeated for a new geographical setting following initial pre-clinical testing, depending upon the similarity of the species in the new setting to those where the antivenom has been formally tested. If species are similar, pre-clinical testing indicates good neutralisation and evidence of clinical efficacy exits from other settings, post marketing surveillance studies may be adequate.

18.2.1 Dose finding studies

Dose-finding studies seek to establish the optimum dose of an antivenom. This would normally be performed as a phase II study, but comparative doses of antivenom may also be studied in large phase III randomized controlled trials (see below). Pre-clinical testing may be used to estimate starting doses and these dose regimens may be evaluated in prospective observational studies (using differing or escalating dose regimens) using standard efficacy and safety

endpoints. Observational studies do not formally randomise participants, but simply carefully record clinical outcomes and reaction rates. Results may sometimes be compared to previous studies (historical controls) to determine how the efficacy or safety of a newly introduced antivenom compares to previously used antivenoms.

18.2.2 Randomised controlled trials

Definitive Phase III randomised controlled trials (RCTs) may require large numbers of patients because of considerable individual variation in the clinical manifestation of antivenom. The new antivenom is compared with the existing standard antivenom treatment or if none exists, two different doses of the test antivenom may be compared. Placebo controls are rarely justified and only if there is genuine uncertainty about the risk/benefit of antivenom treatment where none has been used before. In this situation, as a safeguard against unnecessary morbidity in either treatment group, a restricted sequential plan might be incorporated (Armitage, 1975) which allows evaluation of results as the trial progresses, as in the early trials of therapeutic tetanus antitoxin (Vakil et al, 1979). Randomised controlled trials should be performed when a new antivenom is first developed.

To avoid bias, patients should be randomly allocated to the groups and the study should be blinded, at a minimum to those research personnel who are assessing the clinical response and ideally to both investigators and participants. There should normally be a power calculation of the number of patients required in each trial arm. These power calculations are based on the expected difference in outcome between the treatment groups (if designed to demonstrate superiority of one treatment over another) or predefined limits of the acceptable performance compared to an existing product (if designed to demonstrate that the new antivenom is not worse than existing products [non-inferiority]).

18.2.3 Efficacy endpoints for antivenom trials

The assessment criteria (end points) used for antivenom studies should be predefined and objective. They may be clinical or assessed by laboratory investigations. Common endpoints include mortality, time to restore blood coagulability (assessed by the 20 min whole blood clotting test) (Smalligan et al, 2004) or clinical improvement in neurotoxicity. Immunological data, such as the time to the disappearance of venom antigenaemia may also be used (Otero 2006). Surrogate markers such as platelet count are less suitable as they may be affected by complement activation resulting from antivenom treatment itself. Patients should be observed carefully for long enough to reveal evidence of recurrent envenoming (seen particularly with short half-life Fab antivenoms) (Ariaratnam et al 2001).

18.2.4 Safety endpoints for antivenom trials

Close clinical observation is necessary to detect adverse reactions and accurate reaction rates can only be assessed prospectively. Studies should aim to detect both early adverse events occurring at the time or within 24 hours of antivenom administration (such as anaphylactic reactions and

hypotensive effect from complement activation, fever,.etc.) and late reactions such as serum sickness occurring between 5 and 24 days (such as urticaria, arthralgia, lymphadenopathy, proteinuria, or neuropathy).

18.2.5 Challenges in clinical testing of antivenoms

Several particular features of snakebite make clinical testing of antivenoms challenging. These features include the large variation in the consequences of envenoming in an individual making it necessary to study large number of patients, difficulties in identification of the species responsible for envenoming and the inaccessibility of areas where snakebite is sufficiently common to provide sufficient numbers of patients. Clinical studies may also be expensive particularly if they need to be multicentre with the attendant additional complexity and logistics. However, despite these difficulties, a number of RCTs have been undertaken and published since 1974 (Warrell et al.,1974; Warrell et al., 1986; Cardoso et al.,1993; Jorge et al., 1995; Meyer et al., 1997; Smalligan et al., 2004; Otero et al., 1996;1999;2006).

18.3 POST- MARKETING SURVEILLANCE (Phase IV)

18.3.1 Role

Phase IV studies refer to clinical surveillance studies that occur after market authorization of the product. In view of the difficulty in performing standard clinical trials of antivenom in some settings, this may be the only way to study safety and efficacy of an antivenom in a large number of patients. Therefore, Phase IV studies may be of much greater importance for antivenoms than is the case for other products. A period of active post-licensing surveillance should follow:

i) the introduction of a new antivenom (often a regulatory requirement)

ii) the introduction of an established antivenom into a new geographical area

Although phase IV studies traditionally focus on safety, it is critical that post-marketing studies of antivenoms examine both efficacy and the frequency of immediate or delayed side-effects. The combination of preclinical testing and postmarketting surveillance studies is a minimum acceptable clinical evaluation when an existing antivenom is used in a new region.

18.3.2 Possible approaches

Passive surveillance is currently practised by some antivenom manufacturers. However, approaches that rely upon voluntary return of questionnaires about safety and efficacy are unlikely to provide the high quality data that are necessary. There are two potential approaches to obtaining such data:

a) Countries using antivenoms should establish a national/regional system for the post-marketing surveillance of antivenoms. Clinicians and health workers (such as those working in poison centres) should be encouraged to report actively to national control authorities and manufacturers any unexpected lack of clinical efficacy and adverse reactions. These should include both early adverse events, occurring at the time or within 24 hours of antivenom administration, and, late reactions between 5 and 24 days (see Section 18.2.7). The mechanism for reporting (such as the use of standardized forms), the receiving body (e.g. the national control authority), the deadline for reporting, and the type of adverse events reportable need to be clearly defined by the authority and will depend on its structure and resources. The manufacturer of the antivenom and the authorities should assess these reports and in consultation with each other and with specialists in the field, attempt to evaluate their significance. This assessment may require the testing of products already released and the inspection of production and control facilities and local distribution channels. If an imported product is associated with adverse reactions, the manufacturer and the national control authorities both in the country of distribution and from the country of origin as well as the WHO Global database (WHO f) should be notified.

b) In certain situations for example, the first use of an established antivenom in a new geographical setting or when routine surveillance has identified safety or efficacy concerns, there is a rationale for setting up observational studies to ensure adequate efficacy and safety. In the case of first use of an established antivenom in a new setting, such studies should follow preclinical testing that ensures neutralisation of locally important venoms. Observational studies should carefully document the clinical responses to antivenom (see Section 18), clinical outcomes and the frequency of reactions in a cohort of patients.

18.3.3 Responses to results of post- marketing studies

High quality post-marketing studies will allow clinicians, public health officials and manufacturers to identify poorly effective antivenoms, incorrect use and dosage of antivenoms and serious safety issues arising from the use of antivenoms. In some situations, these issues may be addressed by improving training of staff in the management of snakebite, but these studies may also allow identification of the use of an inappropriate antivenom. (Warrell 2008). Employing sentinel sites is another method of capturing post-marketing surveillance data when other methods prove too difficult.

18.4 Main recommendations

- **Adequate pre-clinical and clinical testing of antivenoms has been largely neglected in the past. Despite challenges, clinical trials of antivenoms in human patients have proved feasible and useful. As far as possible, trials should adhere to the principles of WHO and ICH Good Clinical Practices and should measure robust endpoints**